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# Low levels of second-line drug resistance among multidrug-resistant *Mycobacterium tuberculosis* isolates from Rwanda

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## Summary

**Background:** Multidrug-resistant tuberculosis (MDR-TB) has become a therapeutic problem in many parts of the world, necessitating the inclusion of second-line anti-tuberculosis drugs in specific treatment regimens.

**Methods:** We studied the susceptibility of 69 MDR *Mycobacterium tuberculosis* isolates from Rwanda to second-line drugs by the BACTEC 460 method.

**Results:** The results showed that 62 (89.9%) were resistant to rifabutin while a low rate (4.3%) of resistance was registered for ofloxacin; there was one case (1.4%) of resistance each for *para*-aminosalicylic acid, kanamycin, ethionamide, and clarithromycin.

**Conclusions:** This information is important for devising an appropriate treatment regimen for MDR-TB patients in order to stop the spread of MDR strains and contain the acquisition of additional drug resistance in Rwanda.

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## Introduction

The global rates of tuberculosis (TB) continue to rise, as do rates of drug-resistant TB. Exact rates of drug resistance are unknown, but the World Health Organization/International Union Against TB and Lung Disease (WHO/IUATLD) global

project on anti-TB drug resistance surveillance has conducted over 77 surveys and has noted high rates (>6.5%) of multidrug-resistant (MDR) TB (i.e., strains resistant to at least isoniazid (H) and rifampin (R), the two most potent drugs and mainstay of anti-TB treatment<sup>1</sup>) in some areas of the world, such as Eastern Europe.<sup>2</sup> In resource-poor areas, inconsistent drug supply and weak TB-control infrastructure can lead to a vicious cycle of inadequate treatment and the generation and transmission of MDR-TB strains.<sup>3</sup>

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In Rwanda, TB is one of the leading causes of mortality. In 2003, the annual incidence of new sputum smear-positive cases was estimated to be 161/100 000 population and the prevalence was 664/100 000 population. The TB incidence in Rwanda has doubled over the last decade mainly due to the impact of the human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) epidemic.<sup>4</sup> A recent national survey on drug-resistant TB indicated a relatively high prevalence of MDR with 3.9% among new cases and 9.4% among retreatment cases.<sup>5</sup> However, this survey did not systematically examine resistance to second-line drugs.

Cure rates for MDR-TB patients are low when standard first-line DOTS (directly observed therapy short course) is administered, but with the inclusion of second-line drugs in adapted treatment regimens, treatment success rates vary from 48% to more than 80% of patients being cured or probably cured.<sup>6</sup> MDR-TB patient management is often carried out using the DOTS-Plus strategy,<sup>6</sup> and opportunities to treat MDR-TB in low-resource countries are now available through the Global Fund to Fight AIDS, TB, and Malaria and the Green Light Committee for Access to Second-line Anti-TB Drugs. MDR-TB treatment can be based on the individual results of susceptibility testing to second-line drugs (individualized treatment), or can be standardized, i.e., the same regimen for all MDR-TB patients within a specific TB program.

In vitro susceptibility testing to second-line drugs is not well standardized. In addition, individual testing is difficult and expensive to organize in many settings, and results are only available after a few weeks. Alternatively, a population-based estimation of drug resistance to second-line drugs by analyzing a sample of MDR strains can be the basis for a standardized MDR-TB treatment scheme in a specific region.<sup>7</sup>

In the present study, we aimed at performing second-line drug testing for MDR-TB patients resulting from a convenience sample, in order to provide specific information that can contribute to the development of an empiric second-line treatment regimen for Rwanda.

## Materials and methods

### Study area and population

Rwanda is a small landlocked country in East Africa with 11 provinces and an estimated population of 8.6 million. The National Tuberculosis Control Program (NTP) of Rwanda was created in 1990 and has applied the DOTS strategy since its inception.<sup>4</sup> After the 1994 war, which disorganized the health system completely, the TB program expanded progressively to reach full DOTS coverage in 2000. There are four reference hospitals, 35 district hospitals, and 133 health centers (including nine prison dispensaries) that assure TB detection under the Ministry of Health (MOH). Diagnosis of TB in the NTP depends largely on quality-controlled sputum smear examination. Treatment of TB patients is available in most parts of the country through DOTS. The current treatment regimen is 2(RHZE)<sub>7</sub>/4(RH)<sub>3</sub> for new cases and 2(RHZE)<sub>7</sub>S<sub>7</sub>/1(RHZE)<sub>7</sub>/5(RHE)<sub>3</sub> for retreatment cases (R = rifampin, H = isoniazid, Z = pyrazinamide, E = ethambutol, and S = streptomycin; numbers before the letters indicate the duration in months

of the phase of treatment; subscript numbers indicate the number of times the drug is taken each week).<sup>4</sup>

### Sampling of MDR-TB

This study was undertaken prospectively in seven diagnostic centers in four cities in Rwanda, i.e., Kigali with the highest incidence (331.3/100 000), followed by Butare (129/100 000), Kibungo (80/100 000), and Ruhengeri (23.9/100 000).<sup>4</sup> This study was approved by the Ethics Review Board at Centre Hospitalier Universitaire de Kigali (CHUK), Rwanda.

From September 2002 to March 2005, all smear-positive pulmonary TB patients were systematically included in the study, and a standard WHO questionnaire was used to discern the history of disease, the results of HIV serology, and demographic data.<sup>5</sup> Categorizing of new and previously-treated patients was ensured by strict adherence to the standard WHO/IUATLD definitions and by cross-checking with the patient treatment records where available.<sup>1</sup>

Drug-susceptibility testing (DST) identified 69 MDR-TB cases (35 new and 34 previously treated cases) among 710 smear-positive pulmonary TB patients from these four settings. New patients were defined as patients who had never received anti-TB drugs or had received them for less than 1 month. Relapse was defined as recurrence of disease in a patient previously treated for TB who had been declared cured or who had completed treatment prior to becoming smear-positive again. Failure was defined as a sputum-positive finding in a patient who had been receiving treatment for at least 5 months.

### Identification of strains and drug-susceptibility testing

All sputum samples were mixed with 1% cetylpyridinium chloride (CPC) as transportation medium. The samples were collected on a weekly basis from health districts and transported to the National Reference Laboratory (NRL) in Kigali for analysis. Every sputum sample was accompanied by a shipment form that contained information on the date of sputum collection, sample number, treatment history of the case (new or retreatment), and the quantified result of microscopy examination. Upon receipt at the NRL, each sample was cultured on Löwenstein–Jensen (LJ) medium after decontamination using the Petroff procedure.<sup>8</sup> The cultures were incubated at 37 °C and read weekly for growth for a maximum duration of 10 weeks. Primary cultures that resembled *Mycobacterium tuberculosis* were sent to the Microbiology Laboratory, St Pierre Hospital, Belgium for species identification and DST.

All clinical isolates were identified as *M. tuberculosis* using standard microbiological tests and the 16S rRNA hybridization technique (AccuProbe; Gen Probe, San Diego, CA, USA).<sup>9</sup>

DST to the first-line anti-TB drugs was performed by the radiometric BACTEC 460 method (Becton Diagnostic Systems, Sparks, MD, USA) (E 5 µg/ml; H 0.2 µg/ml; R 2 µg/ml, and S 4 µg/ml).<sup>8,10</sup> The isolates were stored at –70 °C, and all MDR isolates were inoculated on LJ medium and cultured at 37 °C for 3 weeks prior to second-line drug testing by BACTEC

**Table 1** Drug resistance patterns to second-line drugs among MDR-TB isolates according to case category. Susceptibility was determined using the BACTEC 460 method

Second-line drug	Number of resistant isolates according to patient category (%)			Total number of resistant isolates (%)
	New cases	Previously treated cases		
		Relapse	Failure	
Kanamycin	1 (2.85)	0	0	1 (1.4)
Clarithromycin	0	0	1 (3.6)	1 (1.4)
Ethionamide	1 (2.85)	0	0	1 (1.4)
PAS	0	0	1 (3.6)	1 (1.4)
Ofloxacin	1 (2.85)	1 (16.7)	1 (3.6)	3 (4.3)
Rifabutin	32 (91.4)	5 (83.3)	25 (89.3)	62 (89.9)
Total	35	6	28	69

MDR-TB, multidrug-resistant tuberculosis (resistance to at least isoniazid and rifampin); PAS, *para*-aminosalicylic acid.

460 using the following concentrations: kanamycin (KM) 20 µg/ml, rifabutin (RFB) 1 µg/ml, *para*-aminosalicylic acid (PAS) 0.5 µg/ml, clarithromycin (CLA) 4 µg/ml, ethionamide (ETH) 2.5 µg/ml, and ofloxacin (OFX) 1 µg/ml. The choice of the drugs was based on the practical needs of the TB control program in Rwanda; these drugs are those planned for use in the treatment of patients with MDR-TB. Drug concentrations were adapted from Walter and Heilmeyer<sup>11</sup> for the BACTEC 460 method based on the mean maximum blood levels that can be reached in a patient and on data obtained with specific drug-resistant reference strains. External quality assessment organized by the Pasteur Institute of Brussels yielded sensitivities and specificities of 100% respectively for ETH, OFX, KM, and RFB. Internal quality control of first-line DST was ensured by testing 15 MDR-TB isolates on two different occasions, and external quality control by participating biannually in a national quality control organized by the Pasteur Institute of Brussels. Results of internal identification and DST were concordant for all isolates, and a 100% specificity and sensitivity were obtained for H, R, and E in the external assessment.

## Data management and analysis

Data were double-entered using the Statistical Package for Social Sciences (SPSS version 11.5) software on a weekly basis during the inclusion period. These data were later linked with DST results from the St Pierre laboratory. Both data sets were compared using EpiInfo version 6.04d and cleaned by verifying the paper-based questionnaires, sample-transportation forms, and DST results. Analysis was done according to WHO/IUATLD recommendations. A *p* value <0.05 was considered significant.

## Results

From January 2002 to September 2005, all notified smear-positive (new and retreatment) cases were enrolled in the study and followed-up for treatment outcome. Results of first-line drug resistance have been previously published,<sup>12</sup> and will thus not be discussed here. In summary, a total of 710 cases resident in the four epidemiological field sites were notified and 644 (90.7%) of them yielded a positive culture

and valid DST results. Four hundred and eighty-three (75%) were new TB cases while 161 (25%) were previously treated TB cases. The majority (56.8%) were males giving a sex ratio of 1.3:1. The mean age of the patients was  $32.1 \pm 11.2$  years.

Phenotypic DST classified 514 (79.8%) as being pan-susceptible to first-line anti-TB drugs, whereas 55 (8.5%) were resistant to one or more drugs but not MDR, and 75 (11.6%) were MDR. The MDR-TB rate was 7% among new cases and 25.5% in retreated cases.

After storage at  $-70^{\circ}\text{C}$  and subsequent sub-culturing, only 69 of 75 MDR-TB isolates (with 35 strains from new cases and 34 from retreatment patients) were available for second-line DST. General susceptibility patterns according to the case categories are presented in Table 1. Resistance to RFB was high (89.9%), both in new (91.4%) and retreatment (88.2%) cases. Ofloxacin resistance reached 4.3% among all MDR-TB cases, and even 16.7% among relapse cases, although this represents only a single case. Resistance to KM, PAS, ETH, and CLA was very low (1.4% each).

Combined resistance patterns to first- and second-line drugs per patient are presented in Table 2. Seven (10.1%) MDR-TB cases showed no resistance to second-line drugs, 57 (82.6%) showed resistance to a single second-line drug (RFB), whereas five (7.2%) MDR-TB patients showed resistance to two or more second-line drugs (including RFB).

**Table 2** Breakdown of first-line and second-line drug-resistance patterns for 69 MDR-TB

Pattern of resistance to first- and second-line drugs	Number of isolates (%)
HR	7 (10.1)
HRE + RFB	5 (7.2)
HRS + RFB	5 (7.2)
HRES + RFB	47 (68.1)
HRES + RFB + OFX	3 (4.3)
HRES + RFB + CLA + PAS	1 (1.5)
HRES + RFB + KM + ETH	1 (1.5)

H, isoniazid; R, rifampin; E, ethambutol; S, streptomycin; RFB, rifabutin; OFX, ofloxacin; KM, kanamycin; PAS, *para*-aminosalicylic acid; CLA, clarithromycin; ETH, ethionamide.

Simultaneous resistance to all second-line drugs tested was not observed.

## Discussion

The present study on second-line drug resistance among MDR-TB isolates in four regions of Rwanda was initiated out of practical necessity. The current relatively high burden of MDR-TB in Rwanda necessitates the development of an effective second-line drug treatment regimen. Data on second-line drug resistance are useful in devising an effective treatment regimen as this permits the selection of drugs with a low resistance rate in order to obtain good cure rates and avoid acquisition of additional resistance to second-line drugs.

Overall, results were encouraging in that all patients were susceptible to at least two of the different second-line drugs tested.

Resistance to RFB was present in 89.9% of the MDR-TB isolates, reflecting the expected proportion of cross-resistance with rifampin.<sup>6</sup> Therefore, the use of RFB in a standardized MDR-TB regimen would be unwise in cases of known or highly suspected MDR-TB and in the absence of individualized and highly controlled *in vitro* testing. The use of RFB is also likely to be confined to patients co-infected with HIV who are receiving protease inhibitors. HIV-positive individuals in Rwanda have access to free antiretroviral treatment with the assistance of the Global Fund to Fight AIDS, TB, and Malaria.

Apart from RFB, second-line drug resistance was low (KM, ETH, PAS, and CLA). Resistance to fluoroquinolones as determined by OFX was moderate (4.3%), at a concentration of 1.0 µg/ml. This was similar to the result obtained for the recent national survey comprising 701 *M. tuberculosis* isolates in Rwanda (data not shown). One of the three OFX-resistant MDR-TB patients was newly diagnosed, another was a relapse case, and one patient was a treatment failure (Table 1).

Although nearly half (47.2%) of all isolates were resistant to streptomycin, the vast majority (98.6%) of MDR-TB isolates from our study were sensitive to the second-line injectable aminoglycosides. The same observation was made in Russia,<sup>7</sup> and most probably can be explained by a difference in drug targets. All aminoglycosides act by impairing protein synthesis at bacterial ribosome. Half of known streptomycin-resistant mutations are located in the *rpsL* gene encoding for the 12S rRNA and to a lesser extent in the 16S rRNA gene at two conserved loop domains (around nucleotides 530 and 912).<sup>13</sup> Resistance-conferring mutations for amikacin and KM, are also located in the 16S rRNA gene but around nucleotide 1400.<sup>13</sup>

With regard to combined second-line drug-resistance profiles per patient, the vast majority (91.3%) of MDR-TB cases showed no resistance to the second-line drugs tested or only resistance to RFB (81.2%). If you disregard the data on RFB resistance, three cases showed second-line drug-resistance to OFX only, one case to CLA and PAS, and one to KM and ETH. No case of extensively drug-resistant TB (XDR-TB) was detected, if defined as MDR-TB showing resistance to three classes of second-line drugs (Centers for Disease Control and Prevention<sup>14</sup>), or if defined as MDR-TB showing resistance to a fluoroquinolone and an injectable drug (WHO<sup>15</sup>).

Despite the WHO guidelines for second-line drug testing (WHO/CDS/TB/2001.288),<sup>16</sup> standard recommendations on

the drug concentrations to be used for the various methods and media currently applied for DST of *M. tuberculosis* are still lacking. Some inter-laboratory studies have made medium-specific suggestions (Pfyffer et al.,<sup>17</sup> Rusch-Gerdes et al.<sup>18</sup>), but data are still scattered and international organized external quality assessment is scarce. At the moment we started this study, we decided to use the second-line drug concentrations that were installed and validated within the laboratory at that time. For KM and RFB our concentrations are above those recommended by Pfyffer et al. for BACTEC 460 and by Rusch-Gerdes et al. for the comparable BACTEC MGIT 960 system (20.0 vs. 5.0 µg/ml for KM; 1 vs. 0.5 µg/ml for RFB).<sup>17,18</sup> Therefore, our results could represent an underestimation of resistance, especially the low level resistance observed with KM. However, these drugs are not freely available in Rwanda and are not commonly used for treatment of diseases other than TB, making acquired resistance to these drugs unlikely. For ETH, our concentration (2.5 µg/ml) was in between the previously recommended concentration of 1.25 µg/ml for BACTEC 460<sup>17</sup> and the recently recommended concentration in BACTEC MGIT 960 (5.0 µg/ml).<sup>18</sup> For OFX our concentration was half of that recommended (1.0 vs. 2.0 µg/ml) with a possible risk of overestimating the drug resistance rate.

No significant difference in resistance rates was found between new and previously treated cases, indicating that the problem of resistance to second-line drugs is related both to the spread of resistant strains and to the development of resistance to these drugs during treatment, as evidenced by the development of OFX resistance after inadequate treatment (monotherapy).<sup>12</sup>

Most MDR-TB treatment programs have used individualized regimens based on DST results. This strategy requires ready access to reliable laboratory facilities and medical specialists to integrate results and to prescribe tailored regimens. Such resources are not available in many low-income countries. An alternative strategy could involve treating all MDR-TB with a standardized regimen based on common DST profiles of prevalent MDR-TB strains.<sup>19</sup> Our data support a standardized approach of reasonable alternatives to individualized treatment of MDR-TB in Rwanda. Compared to the national survey, our convenience sample detected a higher rate of MDR-TB cases (11.6% vs. 4.6% among all cases), most probably due to the fact that we included more urban areas. The over-representation of MDR-TB cases in this study, however, most probably does not affect our finding of low second-line drug resistance among MDR-TB, as resistance is expected to be higher in urban settings where drugs in general are more easily accessible.

Finally, in order to determine the value of rapid diagnosis of MDR-TB and the relative merits of standardized regimens compared to individualized treatment regimens for treating MDR-TB in low-income countries such as Rwanda and the Great Lakes Region, further prospective clinical trials are needed. This is particularly important in the context of increasing TB/HIV co-infection.

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